CHIROPTICAL ANALYTICAL METHODS

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INTRODUCTION

The word chiroptical is descriptive of the techniques that use optical detection devices that are selective toward optically active (chiral) materials and/or molecules. They are used for structural investigation and analytical determination. There are three chiroptical techniques:

1. Polarimetry, which deals with the angular rotation of plane polarized light, usually at a single wavelength.
2. Optical rotatory dispersion (ORD), in which the angular rotation of the plane polarized light is measured as a function of the wavelength.
3. Circular dichroism (CD), in which the angular rotation is measured as a function of wavelength, but the light is circularly polarized.

Absorption of light energy is not essential to either polarimetry or ORD. It is, however, an integral part of the CD phenomenon making this method the most selective detector for chiral substrates.

THEORY

Theories of optical activity are described in detail in a number of studies (1–6). The physical phenomenon was first observed during experimental investigations of the transmission of solar radiation through Iceland spar, a natural form of $CaCO_3$, by the French astronomer Arago. One year later, Biot (1812) was the first to demonstrate that solutions of certain organic compounds also rotate a beam of incident polarized light. Biot and Fresnel, working independently (1817), reported that the rotatory power of a substance increases as the wavelength is decreased, the phenomenon now called ORD. By 1846, Haidinger had reported differences in the measured absorptions of left and right circularly polarized light, which is the origin of CD.

The first experimental interpretation of the physical basis for optical activity was provided by Pasteur, who observed the hemihedrism of tartrate crystals, which was visually manifest by tetrahedral facets oriented either right or left with respect to the main crystal surfaces for two crystalline forms. His observation that a linearly polarized beam of light was rotated in opposite angular directions by aqueous solutions prepared from the separated crystal forms demonstrated the first direct connection between macroscopic and microscopic, or molecular, asymmetry.

The first theoretical model of optical activity was proposed by Drude in 1896. It postulates that charged particles (i.e., electrons), if present in a dissymmetric environment, are constrained to move in a helical path. Optical activity was a physical consequence of the interaction between electromagnetic radiation and the helical electronic field. Early theoretical attempts to combine molecular geometric models, such as the tetrahedral carbon atom, with the physical model of Drude were based on the use of coupled oscillators and molecular polarizabilities to explain optical activity. All subsequent quantum mechanical approaches were, and still are, based on perturbation theory. Most theoretical treatments are really semiclassical because quantum theories require so many simplifications and assumptions that their practical applications are limited to the point that there is still no comprehensive theory that allows for the predetermination of the sign and magnitude of molecular optical activity.

A chiral substance is defined by the International Union of Pure and Applied Chemistry (IUPAC) as one that interacts differently with left and right circularly polarized light. Two types of molecular optical activity are recognized: inherent dissymmetry characterized by large rotational strengths and inherently symmetrical, but asymmetrically perturbed, molecules for which rotational strengths are less by a factor of a thousand or so.

The first group is characterized by the absence of a plane of symmetry in the molecule, e.g., hexahelicine. The latter type requires the existence of a chromophore in close proximity to an asymmetric center, such as a carbonyl group adjacent to an asymmetric carbon atom.

Polarimetry

Unpolarized light is thought to consist of an infinite number of time-dependent electric and magnetic fields that vibrate in

phase and at right angles to each other in planes that are orthogonal to the axis of propagation. Only the electric vector is considered in theoretical discussions of optical activity. Linearly polarized light is represented by only one of these planes and is given by the vector sum of two in-phase components of equal intensity that are circularly polarized in opposite directional senses (Fig. 1). The components actually propagate in a helical manner with time; however, the polarization projection on the plane, which is orthogonal to the axis of propagation, is circular, thus, the acquired description of light as circularly polarized.

As linearly polarized light is transmitted through an achiral medium, a single refractive index η is seen by both circularly polarized components, and their rates of propagation are equal. The result is that the vector sum is always a linearly polarized beam oriented along the direction of the incident beam, OO′ in Fig. 1a. In contrast, because of the distinctly different interactions that occur between the two helical electromagnetic fields and the helical electronic motion in a chiral medium, two different refractive indices, η_L and η_R, are presented to the coherent beam (birefringence). There being two refractive indices, the left and right components propagate out of phase. On summing the vectors for the transmitted beam, represented for instance by the diagonal of a parallelogram for which OL and OR are adjacent sites, the polarization is still linear; however, a net rotation from the incident direction by an angle equal to α will have occurred (Fig. 1b). Rotational strengths are equal and opposite for optically active molecular or mirror-image pairs (enantiomers) of equal purity.

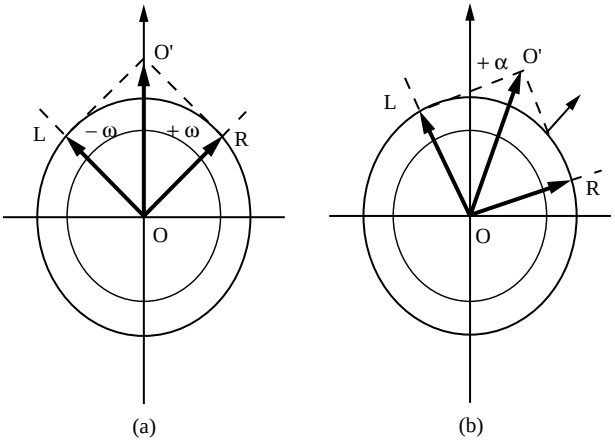

Fig. 1 The incident linearly polarized light OO′ is composed of left and right linearly polarized rotating components OL and OR of equal length. In a chiral medium (a), the rotations are in phase, and the result of the two components is always in the same plane as the incident light. In a chiral medium (b), the components rotate out of phase. The resultant, represented by the diagonal of the parallelogram OLO′R, is rotated through the angle $+\alpha$ from the incident plane.

The magnitude of the optical rotation α (in degrees) is directly proportional to the refractive index difference and to the sample pathlength d, indicative of the fact that rotation is an extensive property, as shown in Eq. 1:

$$\begin{aligned}\alpha &= (\pi d/\lambda)(\eta_L - \eta_R)(1800/\pi) \\ &= (1800d/\lambda)(\eta_L - \eta_R)\end{aligned} \quad (1)$$

and inversely proportional to the wavelength, in keeping with the observed increase in rotatory power with decreasing wavelength. The quantity $(1800/\pi)$ is included to convert radians to degrees. The magnitude of the birefringence $(\eta_L - \eta_R)$ that produces an angle of rotation equal to 1.0° at the Na–D line (590 nm), for a sample with a 10-cm pathlength, is only 3.2×10^{-8}. Because refractive indices are typically approximately 1.0, it is obvious that the absolute size of the birefringence effect is extremely small.

To normalize rotational values when comparing solutions of different concentrations, the specific rotation $[\alpha] = \alpha/c'd$ was defined, where c' was expressed in g/cm^3. This unit is an improper choice for making comparisons among substances with different molar masses M, and therefore $[\alpha]$ was replaced by the molecular rotation term $[\Phi] = [\alpha]$, M/100. In the older literature $[\Phi]$ was expressed in degrees × cm^2/decimole. Division by 100 had no physical meaning whatsoever and was introduced only to keep numbers small. IUPAC has determined that the term molecular rotation is improper and recommended that it be replaced with the more accurately descriptive molar rotation.

OPTICAL ROTATORY DISPERSION

Two types of ORD were first described in 1852 by Biot. In his earliest quantitative experiments on quartz, he demonstrated that the optical rotatory power α varies inversely with the square of the wavelength: $\alpha = \kappa/\lambda^2$. Measurements on a large number of chiral organic compounds, dissolved in solvents both chemically and optically inactive, showed that most of these appeared to obey this law. Originally referred to as the "orthodox" class, these compounds are now thought to produce a plain or normal ORD curve. The distinctive property of the plain curve is that it is always concave to the $\alpha = 0$-axis, regardless of whether the dispersion is positive (Fig. 2a) or negative. A substantial number of organic molecules, however, were found that did not appear to obey this law but had enormously large rotational powers compared with plain curves, which were limited to relatively narrow ranges

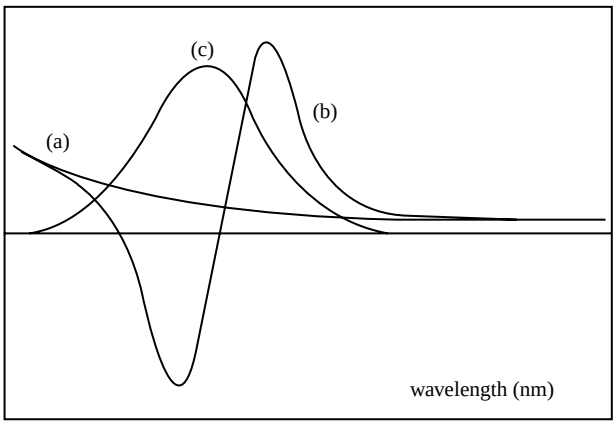

Fig. 2 Composite diagram of the three chiroptical dispersion spectra: (a) A positive plain ORD curve; (b) a positive anomalous ORD curve (Cotton effect); (c) a positive CD curve for a single Cotton effect.

in the spectra. Biot referred to this smaller group as the "heretical" class because of their anomalous behavior (Fig. 2b). Tartaric acid was the seminal example. In an effort to more accurately distinguish between the two types, Lowry specified that for normal ORD behavior, the specific rotation α and its first and second derivatives with respect to wavelength must all maintain the same sign throughout the wavelength range over which the medium is transparent. In other words, it is a mathematical statement to the effect that there should be no zero, no maximum, and no reversal of sign for α as the spectrum is scanned, that is, the curve is always concave to the axis (Fig. 2a).

Among the first experimental discoveries regarding the origins of anomalous dispersion was the observation that the effect could be created by mixing pairs of natural products that generate plain ORD curves, provided they were of opposite signs and unequal rotatory strengths, for example, (l)-turpentine and (d)-camphor. This observation was to be of fundamental importance in the subsequent development of theories for anomalous dispersions. For solutions of tartaric acid, a single pure substance, the existence of an anomalous dispersion was more difficult to explain. It was first assumed that an equilibrium mixture of two molecular forms that generate plain curves of opposite signs must exist in solution. Eventually, however, the effect was correctly interpreted as being a consequence of the molecule having two asymmetric centers that give rise to the "required" unequal and opposite rotations.

The development of several mathematical models and interpretations followed, with the best interpretation being proposed by Drude in Eq. 2, where $i = 1,2,3\ldots$ m:

$$\alpha = \Sigma_I \{k_i/\lambda^2 - \lambda_i^2\} \tag{2}$$

In modern terms, this is rewritten using molar rotational values by replacing α with $[\Phi]$ and k_i with A_i. Drude originally referred to λ_i as the "characteristic vibrational" wavelength, meaning that there were periods of vibration of the charged particles that, when close to the vibrational period of the incident light, would produce the anomalous effect. Again, in modern terms, these are identified with wavelengths of maximum absorbance in the electronic absorption spectra. Whenever $\lambda > \lambda_i$, that is, the wavelength of observation is outside the range of an absorption band, the Drude equation is reduced to the one-term Biot expression for a plain curve. As the value of λ approaches λ_i, α increases asymptotically, reaching infinity at $\lambda = \lambda_1$. Immediately past the maximum wavelength, α is numerically close to minus infinity, and as λ continues to decrease, the curve follows along an inverse asymptotic path towards zero (Fig. 2b).

The anomalous positive ORD curve in Fig. 2b is rounded at finite values for the maximum (peak) and minimum (trough) extremities. The crossover wavelength, where $\alpha = 0$, generally coincides with the wavelength of the maximum absorbance. An anomalous curve is always superimposed on a fundamental plain curve that is alluded to as the background rotation. Media confirmed to have just a single anomalous dispersion can be solved for λ_i. Historically, this procedure was used to predict the wavelength maximum for an incomplete absorbance band that could not be observed in its entirety because of instrumental limitations. This particular application of ORD is now obsolete.

An anomalous curve is referred to as a Cotton effect in honor of the French physicist Aime Cotton (7) who, in 1892, was the first to point out that the absorption of light energy was the other physical property behind anomalous ORD and CD. Positive and negative anomalous dispersions are equally evident in practice. In ORD, the sign of a Cotton effect, by convention, is defined to be positive when the peak precedes the trough as the wavelength decreases and vice versa. A simple structural way of looking at the origins of ORD in a single molecule is to imagine that the fixed asymmetry of a saturated chiral group induces a degree of dissymmetry into an unsaturated and therefore symmetrical functional group or chromophore. In theory $\sigma-\sigma^*$ electronic excitations are possible for saturated molecules for which the theoretical limit of λ_i was determined to be $\approx$150 nm. Chromophores, on the other hand, absorb at much longer wavelengths in the easily accessible range of modern instrumentation. The closer 150 nm is approached, the harder it is to measure a spectrum. Whenever the induced dissymmetry is opposite in sign to the fixed asymmetry, anomalous dispersion is produced. The mutual proximity of the asymmetric center

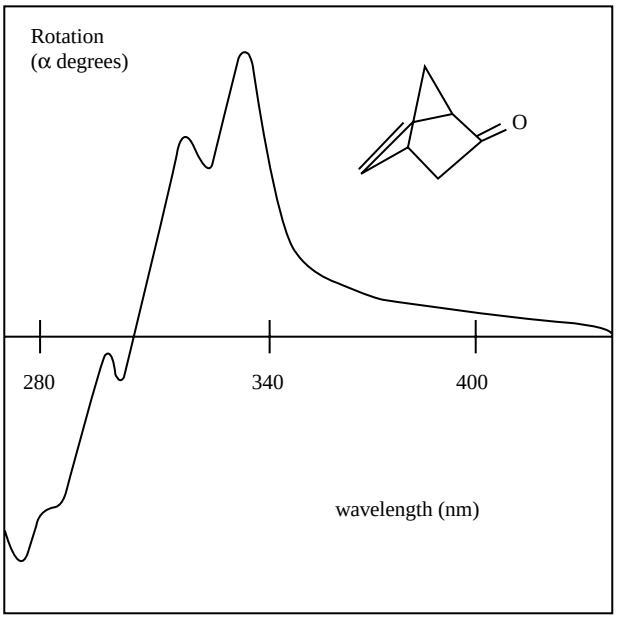

Fig. 3 The anomalous ORD spectrum consisting of several overlapping Cotton bands of the unsaturated rigid ketone, bicyclo(2,2,1)hept-5-enone, which shows how complex the spectrum can be, even for a single molecule.

and the chromophore is a necessary prerequisite to CD induction. Dissymmetry is the preferred description for the induced chirality because the chromophore might well have a high degree of axial symmetry. How this perturbation might occur has never been satisfactorily explained.

Fig. 2b is an idealized illustration of a single, uncomplicated Cotton effect. In reality, the occurrence of a complete curve in the electronic spectrum is rare. Complete dispersions are more likely to be observed in the vibrational spectral range because of the increased spectral resolution. However, even there, dispersions are too often complicated by extensive band overlap. The same is true for electronic spectra where hidden absorption bands coupled vibronic excitations and interferences from bands associated with other chiral chromophores contribute to producing anomalous ORD curves that are so complex they have little utility in quantitative analytical applications (Fig. 3).

CIRCULAR DICHROISM

Because absorption is a prerequisite to CD activity, the phenomenon is limited to only those wavelength ranges that encompass an absorption band in any part of the electromagnetic spectrum. Outside the range of absorption, the CD signal is zero, which is the first important advantage CD has over ORD as an analytical detector. It should be emphasized, however, that the absence of a band is not evidence of the lack of chirality in the substrate.

At the time that Cotton was correctly interpreting the physical origins of anomalous ORD behavior, he proposed that there is also a difference between the absolute absorbances of the two circular polarized beams by a chiral medium (dichroism) and that the magnitude of the dichroism is proportional to the absorbance difference. Convention has dictated that the difference is always written as the absorbance of the left rotating beam minus the absorbance of the right, $\Delta A = A_L - A_R \neq \mathrm{O}$. Using the Beer–Lambert law to convert A to molar units, the dichroism expression can be rewritten as $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$, where ε has the units of L/mol cm.

A single positive CD band is shown superimposed on the anomalous ORD spectrum in Fig. 2c. The wavelength of the maximum CD coincides with the crossover wavelength of the ORD dispersion. Shorter lengths for the vectors OL and OR compared with the incident vector in Fig. 1 are used to convey the fact that absorption has occurred. The absorbance difference at a given wavelength is then represented by unequal vector lengths OL ≠ OR. The resultant of OL and OR, given by the instantaneous diagonal vector OO′ of the parallelogram OLO′R, no longer oscillates in a single plane, but traces out the perimeter of an ellipse as OL and OR rotate around an angle 2π. The transmitted beam is rotated by the angle α from the original plane of polarization (owing to birefringence) and is elliptically polarized owing to dichroism (Fig. 4).

The eccentricity of the elliptically polarized light is characterized by the term ellipticity Ψ equal to the arctangent of the ratio of the minor to the major axis of the ellipse and given by OA/OB in Fig. 4. Because the ratio $(\Delta\varepsilon/\varepsilon)$ necessary to produce an observable CD signal is as small as 1 part in 10^7, the ellipticity is approximated almost exactly by the expression $\Psi = \pi(\varepsilon_L - \varepsilon_R)/\lambda$, which is entirely analogous to Fresnel's equation that relates birefringence to α and points up the common origins of anomalous ORD and CD.

In keeping with the older definitions of terms that are part of polarimetry, there are definitions for specific ellipticity $[\Psi] = \Psi.c'.d$, and molecular ellipticity $[\Theta] = [\Psi]\ M/100$, where M is the molar mass. With appropriate substitutions, the molecular ellipticity can be expressed in terms of e, namely $[\Theta] = 3300(\varepsilon_L - \varepsilon_R) = 3300\Delta\varepsilon$. The numerical constant is the result of all the physical conversion factors. The survival of these arcane units is a

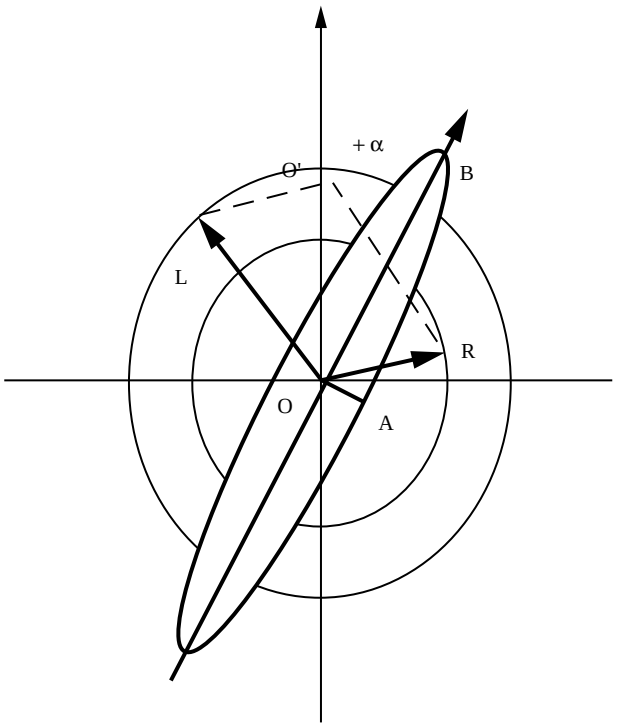

Fig. 4 Production of elliptically polarized light in CD. The direction of polarization of the incident beam is OO′. The unequal vectors OL and OR represent the difference in absorbances of the left and right rotating component beams. The angular rotation is $+\alpha$, and the ellipticity is the arctan (OA/OB).

consequence of the wealth of informational data already in the literature. The disclosure that CD is no more than a modified absorbance technique should ultimately motivate investigators to adopt the term molar ellipticity, Θ_M, in the CD analog of the Beer–Lambert law, that is, $y = \Psi = \Theta\ M\ c\ d$.

Anomalous ORD and CD both originate from light absorption by a chiral species and as such contain the same information. A mathematical equation, the Kronig–Kramers transform, relates one to the other over the wavelength range of the absorption, namely, $[\Theta(\lambda)] = -2/\pi \int [\Theta(\lambda')](\lambda'^2/\lambda^2 - \lambda'^2)d\lambda'$. When the appropriate substitutions are made, the equation relating ORD to CD reduces to $\Theta = 40.28\Delta\varepsilon$.

Because all the rules that apply to absorbance detection apply equally well to CD, it is convenient to think of CD as a modified form of absorption spectrophotometry. Spectra are temperature- and pH-dependent; nonlinear correlations of signal versus concentration are commonplace and are produced for the same reasons, such as chemical equilibria, polychromatic radiation, stray light, etc. Fluorescence emission CD (FDCD) spectroscopy is observed whenever an analyte meets all three of the structural prerequisites simultaneously, either intrinsically or extrinsically. Therefore, anyone with experience in absorption and emission spectrophotometries can easily become acquainted with the experimental capabilities of CD. Similarities end there, however, and the differences are what make CD detection unique, especially the enhanced selectivity that arises from the fact that CD bands can be positive or negative in sign. Electronic absorption bands are generally broad and lack the kind of resolution associated with the infrared range. In contrast to visible-UV absorptions, however, exciton coupling can divide CD bands into two sub-bands of opposite sign and unequal intensity separated by a characteristic crossover wavelength where the signal is zero (6), resulting in narrower bands than those given by absorption. Circular dichroism is used most often for the analysis of bulk samples and has seen limited use in liquid chromatography and capillary electrophoresis.

CD activity can be induced into molecules that are either chiral or achiral and is generally referred to as extrinsic CD. For chiral species, intrinsic and extrinsic CD effects are additive. Compared with intrinsic CD, the extent of extrinsic or induced chiroptical effects is small. One way to induce activity is to apply a static magnetic field whose strength is on the order of 10–20 kGauss. Magnetically induced CD (MCD) was originally described by Verdet and correctly interpreted by Faraday in what has become known as the Faraday effect. A magnetic field of sufficient strength splits the degeneracy of the electronic ground and/or excited states (the Zeeman effect), resulting in absorbance differences between the two circularly polarized beams. The effect is entirely general and can be observed in every dielectric substance that transmits light. The magnitude of the effect depends on the relative orientations of the light path and the magnetic field strength and is at maximum when the fields are parallel. For a fixed geometry, the maximum signal is proportional to the sample pathlength and the analyte concentration. Poor sensitivities and even poorer selectivities associated with MORD and MCD make them unacceptable as analytical detectors.

A second way to induce chiroptical behavior is to associate a chiral center on one molecule with a chromophore on another by some aggregation or complexation reaction. If the chiral moiety is CD-inactive, only the resultant complex exhibits CD activity. The intensity of the induced CD signal is determined by two factors: the concentration of the complex that is formed and the magnitude of the induced $\Delta\varepsilon$ term. Magnitudes and selectivities of chemically induced CD are much greater than those of MCD and have correspondingly

higher potential for analytical applications. Typically, the correlations of signal amplitudes with analyte concentrations are nonlinear.

INSTRUMENTATION

The basic instrumental needs for chiroptical methods are virtually the same as for other spectroscopic methods, namely, a stable unpolarized illuminating source of sufficient intensity, a wavelength-selection device, sample holder, and detector; polarizing elements are essential. Because the only parameter measured in polarimetry and ORD is rotation, the polarizing elements are common to both. A monochromatic source, such as an Na or Hg lamp, is all that is required for polarimetry. Deuterium or halogen lamps are of sufficient intensity for ORD, but highly intense (150–450 W) Xe arc lamps are needed for CD.

Polarizing elements are transparent rhombs constructed by joining together two triangular prisms cut from a single crystal of calcite or quartz. The junction between the two parts may be just air or a light-weight balsam cement. The purpose of the junction is to physically separate the ordinary and extraordinary rays of the linearly polarized light beam, allowing only one ray to pass while the other is selectively reflected in a direction at a right angle to the first. Often, the reflected ray is fully absorbed to eliminate any interference with the transmitted ray. An excellent historic account of the assembly of the parts into working polarimeters is given by Lowry (1).

In polarimetry and ORD, the sample is placed between the first polarizing element (the polarizer), which remains fixed, and the second element (the analyzer), which can be rotated about the axis of propagation. Maximum intensity of the transmitted light is observed when the principal axis of the polarizer and analyzer are colinear and exactly parallel. The intensity is zero when they are crossed; that is, when the principal axes are orthogonal to each other. The most accurate way to determine the rotation angle α is to set the polarizer and analyzer in the crossed position using an achiral substrate and to measure the extent to which the analyzer has to be turned to restore the optical null position when the achiral sample is replaced by a chiral substrate.

Optical rotations are temperature-dependent. For the most accurate work, sample cells must be thermostatted. Solution concentrations are typically above 0.2 M for polarimetric detection, and pathlengths range from 1.0 to 100 mm; volumes vary from 0.1 to 50 ml. Because rotations increase in magnitude with decreasing wavelength, the best sensitivities using conventional light sources are achieved in the UV. Accuracies are reported to be on the order of $\pm 0.2\%$ for rotations $>1.0°$. Only the most sophisticated high-sensitivity polarimeters meet the requirements for chromatographic detection. With a stable laser system as the illuminating source, rotations as small as 10^{-10} to 10^{-11} radians can be measured fairly accurately. High sensitivities are critically important in chromatography because concentrations of eluted components are very low, being limited by the retention capacity of the column materials, and because pathlengths, viewed across the eluant exit tubes, are very short (8).

The first commercial ORD spectropolarimeters appeared in the 1950s but are no longer available. The ORD capability is typically offered as an add-on to a CD spectropolarimeter.

At present, technical difficulties associated with scanning chiroptical methods prevent the use of diode-array detection, and therefore wavelength is selected in CD with a scanning double monochromator set-up (Fig. 5). The block diagram for CD differs from ORD instrumentation by the addition of an electro-optic modulator, placed immediately after the linear polarizer, to generate the phase-separated left and right circularly polarized component beams that are the origins of the elliptically polarized light beam. The physical parameter that was measured in the first CD instruments was the ellipticity of the transmitted beam (Fig. 4). Greater accuracy and greatly improved sensitivities are achieved if the absorbance difference is measured, which is the procedure preferred by every contemporary CD instrument manufacturer.

Because of significant losses of radiant power on polarization and transmission through the double monochromator system needed to keep stray light to an absolute minimum, the light sources for CD detection must be intense. As a consequence, instrument compartments are purged with nitrogen to remove ozone that might be produced by the high-intensity radiation of oxygen. The detector is a photomultiplier tube. Wavelength ranges on commercial instruments extend from 180 to 850 nm. Instruments for the vibrational spectroscopy range are still only custom built. The ellipticity, or $\Delta\varepsilon$, scale should be calibrated daily against selected standards. Scale calibration is wavelength-dependent, and whenever the range of study is very broad, the use of more than one standard is recommended. Those most commonly used are androsterone, pantoylactone, (+)-camphor-10-sulfonic acid, and ammonium camphor-10-sulfonate for the near-UV, and alkaline nickel(II) tartrate in the visible.

With absorbance differences on the order of only 1 part in 10^7 for CD activity, the ratio of transmitted intensities for the left and right circularly polarized beams (I_L/I_R)

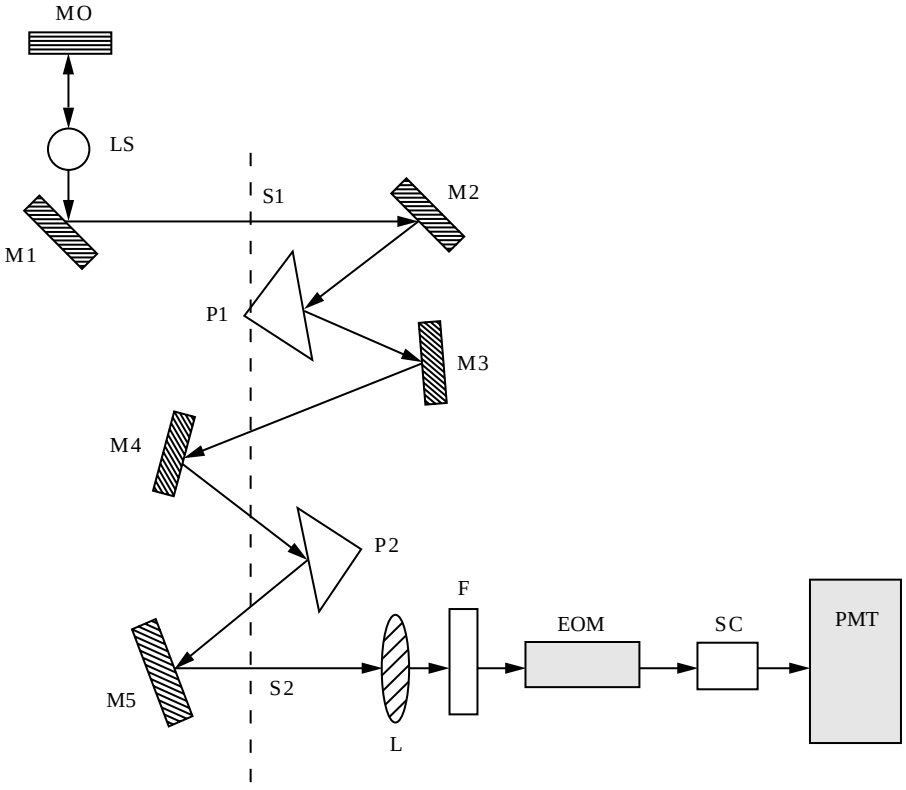

Fig. 5 Block diagram for a commercial double-monochromator CD spectropolarimeter. M = various mirrors; S = slits; P = polarizing elements; LS = light source; SC = sample; PMT = photomultiplier tube; L and F = lens and filter, respectively; EOM = electro-optic modulator.

is essentially one because the errors in $\Delta\varepsilon$ would be very large if I_L and I_R are measured directly.

$$\Delta\varepsilon = (\varepsilon_L - \varepsilon_R) = [(1/c \cdot d)\log(I_L I_R)] \quad (3)$$

The problem is overcome instrumentally by measuring the intensities I_L and I_R separately, at a frequency of 5 kHz. This has the effect of producing an AC voltage proportional to the CD signal, riding on top of a steady-state DC component proportional to the total absorbance at each wavelength. The independence of I_L and I_R from the steady-state DC voltage indicates that the size of a CD signal is not dependent on the absolute magnitude of the absorption, and relatively strong CD signals can be obtained from overall weak absorbers. Although an absorbance difference is measured in CD detection, the total absorbance by the substrate and matrix is still a limiting feature because it can affect the intensities of the transmitted beams to be measured. Excessive amplification of very weak signals increases the noise level and adversely affects the quality of the CD signal.

The principal electronic excitations in the accessible UV range that lead to absorption by organic molecules are the $\pi - \pi^*$ transitions associated with the aromatic ring and the $n - \pi^*$ transitions of carbonyl functional groups. Excitations associated with $\pi - \pi^*$ transitions have a high probability, and absorbances are highly intense. To preserve the signal quality, solutions must be very dilute and/or pathlengths must be short, which is the second advantage that CD has over polarimetry and ORD. The photomultiplier (PMT) measures the total transmitted intensity and is incapable of discriminating between chiral and achiral species. Besides affecting the signal/noise ratio, excessive absorptions reduce the linear dynamic range of the detector. At the low concentration end, the determining factor is the very small size of the CD signal, and the upper limit is determined by the total absorption. Ranges are often much narrower than they normally are using absorption detection.

Current CD instruments commercially available for analytical applications are limited to the electronic excitation range of the electromagnetic spectrum. The more sophisticated of these have the added capability of pulling an eluate from a chromatographic separation off-line into a microcell attachment where, instead of limiting

detection to just one wavelength, a partial CD spectrum can be measured. Volumes can be as small as 10 nL (8). Instrumentation for the measurement of vibrational CD (VCD) and Raman optical activity (ROA) are still custom-built, although the prospects for their commercial development in the not too distant future are bright. A major disadvantage is, of course, that the emission intensities of tunable IR sources are generally weak.

An intriguing recent development in CD detection is its extension to the wavelength range of soft X-Rays using a synchrotron source (9). Although this might never become a routine analytical method, it has been speculated that from CD measurements made in this range, it will at last be possible to indisputably determine the absolute conformation of a chiral molecule of any size in solution. This would make it superior to NMR detection, which is limited to small molecules and single-crystal X-ray structure analyses, in which, for the want of other methods, structures are usually assumed to be the same in solution.

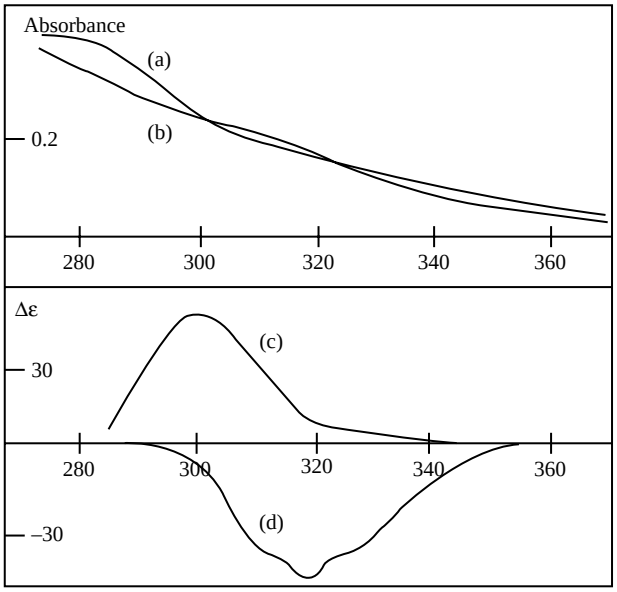

Fig. 6 Demonstration of the strong similarity between the absorbance spectra for (a) dihydrotestosterone and (b) testosterone in methylene chloride in contrast to their CD spectra (c) and (d), respectively.

ANALYTE SELECTION

In deciding whether analytes are CD-active, it is not always a simple matter to inspect a molecular formula and be certain that the chromophore and the chiral center are mutually located in a manner that produces activity. Even if the molecular structure suggests that a chirally perturbed chromophore is present, the substance might only be available as an achiral racemic mixture and therefore is not detected by CD.

Optimum wavelength ranges for CD detection are those where the absorption is minimum and the CD signal maximum. Absorptions for $n - \pi^*$ and $\pi - \pi^*$ transitions are generally weaker at wavelengths longer than 230 nm, where they appear as shoulders on the edges of the intense bands that reach a maximum at shorter wavelengths. Frequently, CD bands in the range of 230–340 nm are intense enough to allow quantitative analysis, e.g., for testosterone and dihydrotestosterone (Fig. 6).

Electronic excitation energies are shifted to longer wavelengths as the extent of molecular conjugation increases. The molecular symmetry that accompanies the structural planarity created by conjugation might reduce the number of potential achiral centers and the chances of observing intrinsic CD activity, for example, in organic dye molecules. Strong absorptions by dyes, however, are exploited by associating them with a chiral molecule to induce an extrinsic CD activity in the longer wavelength range, where passive absorption by the matrix is less of an interference. Other absorbers of this capability are colored chiral metal complexes that have the special advantage that their absorbances are generally of much lower intensity than those of organic dyes.

Similarities between CD and absorbance methods are also found between CD and fluorescence and CD and circularly polarized luminescence (CPL). Three prerequisites are needed to produce FDCD and CPL activities (8). Intense emission signals normally associated with fluorescence are attractive because limits of detection are lowered considerably. FDCD finds more uses as a chromatographic detection device. A CD signal is usually induced by some kind of molecular complexation reaction. Association can be with a simple molecule or with an aggregate of molecules, such as chiral micelles, which are known to be fluorescence enhancers. In cases of color induction combined with fluorescence induction, FDCD can lead to even higher levels of selectivity among analytes that have been derivatized by the same color reagent.

Selectivity enhancement is a result of a number of circumstances. For example, not all of the sub-bands in the absorption or fluorescence spectrum of the derivative are necessarily CD-active; because only the chromophore next to the chiral center must fluoresce for CD activity, all other fluorescence that centers on the analyte, or in the matrix, will not interfere with the FDCD signal, and if a chiral center is lost in the derivatization process, that molecule will be removed from the list of interferences.

CHIROPTICAL DETECTION IN CHROMATOGRAPHY

Only polarimetry and CD find practical use as chromatographic detectors (8). Important parameters to consider in modifying chiroptical detectors for use in chromatography are the very small sample volumes involved, which today can be handled with relative ease, and the very short time intervals that separate consecutive peaks, which has not been, and probably never will be, totally resolved. Peak overlap remains a significant problem. In molar terms, limits of detection are not particularly impressive, for example, micromolar levels, but in volumes as little as 1.0 μl, the limits of detection are actually at the nano- to picomole level and sometimes lower (10–14). Engineering priorities are to develop the technology to focus the beam on such small targets while maintaining the high level of radiance needed for chiroptic detection. CW lasers are an obvious place to start, but source noise and instability are problems to contend with (14). By exploiting the added radiance of pulsed lasers, limits of detection can be stretched to even lower levels. Laser sources, however, are limited in the number of their output wavelengths. Dye lasers offer the best, albeit still very narrow, ranges (approximately 60 nm). Current CD instruments, in which the source is laser illumination, really do operate at just a single wavelength, depriving the detector of its ability to identify an analyte.

Options for multichannel LC–CD detection do exist (14–16). Stopped-flow accessories for commercial instruments are available that allow part of an eluted fraction to be taken off-line into a microcell placed in the regular sample compartment where data are measured in the normal way. The method still requires rapid scanning capabilities. Repeated injections and multiple scans can be averaged to improve the quality of the signal. A major deterrent to the progress in the early development of HPLC–CD detection was the lack of a dedicated instrument at a reasonable cost, the only option being a fully equipped CD instrument.

In 1998, Jasco International Co. introduced the first dedicated commercial polarimetric detector, the OR-990, and the first dedicated CD cum absorbance detector, the CD-1595, for HPLC. The CD detector operates in the 220–420 nm range, with a 20 nm bandwidth. The illuminating source is a 150 W Hg–Xe lamp. Injections are typically in the microgram range. Minimum detectable amounts are on the 0.1-ng scale. The sensitivity of the CD detector is typically 200 times higher than that of the OR-990 detector but of a factor of four lower than the absorbance detector of the CD-1959. The latter is the limiting factor in the combination of CD with absorbance detection when applied to enantiomeric purity measurements. This last assay has taken on considerable new meaning with the obsessive focus on measuring the enantiomeric purities of chiral drugs in the biotechnology and pharmaceutical industries.

Eventually these devices may turn out to be the starting point for the development of CD diode-array detectors. Adjusting the scanning speed for on-line, wide-spectrum CD measurements is a formidable problem. A major reason for the problem is the incongruity between the time it takes to accumulate CD data, even for just one spectral "pass" using the very best currently available diode array technology, and the typical dispersion time between chromatographic peaks. The situation may very well change as faster electronic detection devices become available.

If all the components of a sample loaded on an HPLC column are baseline-separated, any conventional detector will work, unless the object of the separation is to determine or confirm the stereochemical conformation of an enantiomer. In achiral systems, (solvent and/or stationary phase) enantiomers have identical retention times and are not separable. The problem has a solution if two detectors are used in series, e.g., CD and absorbance (17–19). Because the enantiomers elute together, the absorbance detector measures the sum of their concentrations, and the CD detector measures the difference ΔA. Solving the simultaneous equations gives the concentrations for both enantiomers.

Historically, the experimental limitations of this procedure give totally meaningless results when enantiomeric ratios are greater than 95:5, or within 5% of being racemic (20). Concepts that have evolved as potential solutions to these experimental limitations making them capable of improving on the accuracies of enantiomeric purity determinations are the g-factor and principal component analysis (PCA) treatments of eluted band intensities as a function of time (21, 22). These are especially useful in cases in which bands are asymmetrical (22), which is frequent. The *g*-factor is defined as the ratio of the CD intensity to the absorbance intensity $\Delta A/A$. One attribute of this factor is that for an enantiomerically pure material, the g-ratio does not change with concentration. Should a change in the g-factor occur during the elution of a band, it is clear evidence for an enantiomeric impurity. Ideal traces documenting the total resolution of bands for two pure enantiomers in a racemic mixture would consist of two horizontal lines with constant g-values of equal and opposite signs. Over the time interval between the elutions, the traces are separated by signals that are excessively noisy. This occurs because calculated

g-factors in the ranges in which no CD-active species is being eluted correspond to zero divided by zero. With PCA, the number of components that are coeluted can be derived by reduction of a matrix of signal intensities versus concentrations versus time data for a series of solutions with prepared compositions (21).

Ostensibly, the better alternative is to separate the enantiomers on a chiral HPLC system, typically done by reacting both enantiomers of a racemic mixture with a third chiral species. The chiral derivativizing agent is an integral part of either the mobile phase or the stationary phase (19). The products are two diastereoisomer derivatives with different retention times. The same principle was used in classic experiments in which, for example, (−)brucine was added to separate enantiomers by fractional crystallization. It is still the only viable option to discriminate among enantiomeric forms by NMR. The numerous problems associated with chiral chromatographic methods are familiar (20, 21):

- The number of chiral derivatizing agents that are 100% enantiomerically pure is extremely small.
- Differences in retention times are very small if the material has several chiral centers.
- In practice, chiral solvents can be used as mobile phases only once.
- Even if derivatization is accomplished, baseline separation is not guaranteed.
- Racemization of the analyte may occur on the column during elution.

The protocol for separating a partial racemic mixture calls for the chromatographic conditions to be modified in such a way that the minor component elutes first (23) and is not lost in the trailing edge of the band for the major component. Errors encountered in the determination of enantiomeric excesses when they are in the range of 98–100%, or close to unique protocol is required for every chiral analyte assayed by chiral-HPLC, requiring considerable development time and constant review of the procedure.

DIRECT CHIROPTICAL DETECTION

In this context, direct means separation of the substrate, except solvent extraction, is not a part of the analytical work up (24–26). Only CD has the necessary selectivity to function as a direct detector. Chiral molecules that do not absorb (e.g., most simple sugars) do not interfere. Achiral molecules that absorb interfere to the extent that their absorption lowers the signal/noise ratio and the limits of detection. Naturally occurring pigments and coloring agents added to pharmaceuticals are among the worst interferences.

Overlapping bands from multiple CD-active analytes are also a concern, although there is less of a tendency for this to happen with CD compared with absorption because bands are generally narrower and often have opposite signs. Curve-fitting algorithms might be used to resolve overlapping bands, but these kinds of solutions often lead to ambiguous results. More and more attention will be given to pattern-recognition strategies that involve data analyses that use chemometric methods such as principal component analyses and artificial neural networking (27).

Molar ellipticities in the preferred wavelength range of 230 to 340-nm for underivatized analyses typically differ by only a factor of two or three. By comparison, linear dynamic ranges are much greater than this, and the limiting property in discriminating among CD signals is the analyte concentration rather than the rotational strength of the chiral chromophore. Limits of direct CD measurements made on bulk samples using direct transmission detection are similar to those for absorbance, approximately 100 nM for a l.0-cm pathlength.

REFERENCE CD SPECTRA

There are no comprehensive data files for CD spectra for standard reference materials (SRM) that compare with the exhaustive libraries which have been compiled for absorbance data in the electronic and vibrational spectroscopy ranges. Analysts are required to create their own CD spectral files using SRM prepared by the usual purveyors of fine chemicals. A significant problem with an SRM is that although it might meet the industry specifications for chemical purity, its enantiomeric purity is open to question. The few cases in which absolute enantiomeric purity might be assured involve natural products whose syntheses are under total enzymatic control. To prove 100% enantiomeric purity is beyond current capabilities (20). The problem is compounded even more with the risk that the material might racemize after its extraction from its natural environment. Therefore, it is not possible to assume absolute enantiomeric purity with firm conviction.

The superficial observation that CD spectra for enantiomers are exact mirror images of each other is only true if the two SRM used to calibrate the CD have equivalent enantiomeric purities. And even if the spectra are exact images, the evidence is not irrefutable proof that both SRM are 100% enantiomerically pure.

Added complications arise when an analyte molecule has two asymmetric centers for which there are a total of four optical isomers (R,R; R,S; S,R; and S,S). Together they constitute two pairs of diastereoisomers for which there are two pairs of "equivalent" CD spectra. It is conceivable then that the wrong analyte could be identified and assayed. The practical solution of these disconcerting uncertainties is to run regular checks on the reproducibility of the spectrum for a chemically pure SRM that has been "defined" to be enantiomerically pure. This is done by adding spectral data for every new issue of an SRM, supplied from different product lots by different manufacturers, to an ever-increasing data pool and periodically updating the statistically averaged spectrum as the reference spectrum. The inability to get standards of absolute enantiomeric purities takes on an even greater practical significance when attempts are made to assay enantiomeric excesses or enantiomeric purities in mixtures of isomers that may have been produced synthetically.

APPLICATIONS

The ubiquity of the aromatic ring and carbonyl chromophores in the molecular structure of natural products means that the number of potential analyses is enormously large. Djerrasi (2) pointed out the analytical potential of chiroptical methods as long ago as 1960, but even now, the number of investigations is small, which is explained in part by the enormity of the field of separation sciences. Most analysts would argue that the obsession with separation is because problems with interferences are minimized. On the other hand, for many of these processes, their potential was illustrated using carefully chosen synthetic laboratory mixtures, most of which were so simple they did not even begin to address the complexities that are encountered in the analysis of real samples.

The emphases of this section reflect the author's own special interests in using CD detection to directly determine chiral substrates. The majority of the systems described are drug substances. Direct analytical applications over the last 20 or so years have clearly demonstrated that a priori expectations of serious interference problems are ill-founded. Analytical sensitivities similar to those for absorbance spectrophotometry are readily accessible, and a high degree of analytical selectivity is obtained because of that very same property that makes ORD and CD such useful structural tools, namely, the sensitivity of a chromophore to its chiral environment (3, 6). Substrates are organized into three groups:

1. Those with chiral chromophores that absorb in the near-UV
2. Those that are either chiral or achiral but do not absorb and are derivatized to absorb in the visible
3. Those that are achiral and absorb and have optical activity induced by interaction with a chiral host.

CHIRAL CHROMOPHORES THAT ABSORB IN THE NEAR-UV

The major analytes in this category are the alicyclic compounds (alkaloids and terpenes); heterocyclic compounds (barbiturates, benzodiazapams, indole alkaloids, quinolines, nucleic acids, and nucleotides); aminoacids and peptides; oligopeptides; and proteins (globular, nucleo-, and lipo-); saccharides and polysaccharides; and condensation products of saccharides with all the other analytes, e.g., glucuronides and glycoproteins (26). Thus far, most analyses have been done on solid and solution forms of the drug substances. A few illustrations are reported in which CD was used in the direct analysis of biological extracts.

Morphine Alkaloids

The first series of compounds assayed directly by CD detection were the morphine alkaloids. They were supported in aqueous solutions (28), in a chiral cholesteric liquid crystal solvent (29), and mixed in pellet form with solid KBr (30). Contrary to expectations, the homogeneous aqueous solution medium gave the best selectivity among 10 related opiates and the most quantitative results. The pH-dependence of phenol substituted analogs, which in some instances caused the sign of the CD signal to invert, enhanced the selectivity. Heroin was assayed both directly and as the morphine hydrolysate (31). Direct multicomponent analyses were made for prepared mixtures of morphine, codeine, thebaine, noscapine, and opium extracts (32).

Aromatic Amines

The strong structural similarities and the proliferation of enantiomeric forms in the phenethylamine and catecholamine series are major reasons for the considerable difficulties encountered in their analyses (20). Absorbance bands in the 250- to 320-nm range are identified with the aromatic ring and are nondiscriminatory.

Chiroptical detection methods have a slight edge over absorbance and a large advantage over electrochemical detection because of the birefringence factor. An ORD detection assay was developed to analyze mixtures of ephedrine and pseudoephedrine (33). An unprecedented advantage was found in the determinations of amphetamine and methamphetamine in cases in which achiral excipients such as lidocaine, procaine, and benzocaine had been added to deliberately confuse the assay by absorbance detection (34). These additives have absorbance spectra and retention times in achiral liquid chromatography that are too similar to the analytes.

Antibiotics

All the tetracyclines have intense visible CD spectra, with some degree of discrimination among them possible (35). The β-lactam antibiotics have very similar near-UV absorbance spectra, making some kind of separation the method of choice for their determination. Discrimination between the penicillin and cephalothin groups by CD detection, however, turned out to be an elementary exercise when mixtures of Pen-V and cephalothin were simultaneously determined with equal imprecisions in prepared laboratory mixtures (36). In contrast, discriminations among individual members of either β-lactam group is a very difficult prospect that will, in all likelihood, require a prior chemical derivatization. An ORD study reported the discrimination between the neomycin B and C aminoglycoside antibiotics. However, the strong similarities between the absorbance and CD spectra for the polymixin and bacitracin antibiotics (30) make their discriminations by direct assay impractical unless there is first a derivatization step.

Alkaloids

Other alkaloids assayed with varying degrees of success include the quinine–quinidine, cinchonine–cinchonidine, digoxin–digitoxin, L-Hyoscyamine–atropine, and pilocarpine–isopilocarpine diastereoisomeric forms. Being diastereoisomers, these have different chromatographic retention times, yet their assays are confused when compositions of the enantiomeric mixtures change. Prepared binary mixtures of the first two pairs of diastereoisomers were easily quantified using direct CD detection (37). Observed signals for the digoxins and pilocarpines, on the other hand, are so weak that the best possible analysis was qualitative identification. The CD spectra for the colchicine, strychnine, brucine, and tubocurarine alkaloids, all potent poisons, have been characterized in strong aqueous acids (38, 39). No reports of their being assayed by chiroptical methods have appeared.

Vitamins

In the area of vitamin analyses, CD spectra have been characterized for the water-soluble vitamins B2 and C. When they occur together, their distinction by direct measurement is an elementary procedure. Both were successfully assayed in the extracts of pharmaceutical preparation, as was B12 (40). Analysis of the fat-soluble D2 and D3 vitamins (ergocalciferol and cholecalciferol) has not been equally successful (38). Vitamin D extracted from natural sources has a single conformational stereochemistry that is one of several isomers produced in synthetic preparations. To certify that the natural form is present in a synthetic product, where it can be accurately assayed in the presence of the other isomers, is a formidable analytical task. Whether direct CD detection can satisfactorily solve it is currently unknown. A prior nonselective derivatization reaction might be required on all isomers. The A and E vitamins are achiral and not subject to chiroptical detection unless first derivatized by reaction with a chiral host.

Steroids

The seminal work on steroid analyses using chiroptical detection was done by Djerrasi by the determination of hecogenin acetate in the presence of tigonenin acetate (2). Every steroid is chiral and therefore amenable to polarimetric detection after chromatographic separation. Chromophores are fairly uncommon, and analysis by ORD or CD is therefore less suitable. The only unsaturation in the cholesterol molecule, for example, is the isolated Δ^5-double bond, which has an absorbance maximum at 205 nm. Unsaturation coupled with chirality provides some selectivity, as ably demonstrated by the work of Potapov for analogs of progesterone (41). Even simpler than that is the direct discrimination between the ketosteroids testosterone and dihydrotestosterone, which have opposite signs in methylene chloride solution (Fig. 6).

Gergely promoted the development of ORD methods for the Δ^4-3-Ketosteroids and the 17-Keto- and 17-Ethynyl derivatives (42). The 17-Keto derivative is often present as an impurity in the manufacture of 17-Ethynyl-substituted steroids and is easily quantitated by mathematically fitting the spectrum for the mixture using weighted spectra for the components or by measuring the spectra in two solvents. In the second option, data at two wavelengths are used to

prepare simultaneous equations that are solved for the concentrations of both components. The latter was used to quantitate mixtures of corticosteroids and Δ^4-3-Ketosteroids (42). For the most accurate results, however, chemometrics methods are recommended with full spectral data.

Carbohydrates

Although included in this subsection, the only carbohydrates that meet the condition of absorbing in the near-UV are the keto-, amido-, and carboxylate-substituted sugars (43). Fully saturated sugars absorb only at wavelengths less than 200 nm and in general have incompletely developed spectra, with many not reaching a maximum signal. In the near-UV, excellent analytical data have been obtained for the in situ determination of D-fructose in honey (44) and of the *N*-Acetyl content of chitosan in crustacean shells (45). Simple sugars commonly exist in the form of equilibrium mixtures of open-chain and cyclic anomers (46), in which equilibrium must be established and the temperature controlled for the most accurate and reproducible measurements. Aldoses are typically determined by high-performance liquid chromatography (HPLC) using absorbance detection at approximately 300 nm. Polarimetry can be, and has been, used whenever information on the enantiomeric forms of the eluants is needed. Kuo and Yeung combined both these detectors for the analysis of several saccharides in laboratory mixtures. An advantage is that the focus is narrowed to cover only chiral absorbers, which simplifies the analytical identification of the eluates. Of the systems in which HPLC with CD detection was used (47), the most common are the simplest ketoses, D-fructose, D-tagatose, D-sorbose, turanose, D-ribose, and vitamin C.

Aminoacids, Peptides, and Proteins

Most of the findings related to CD detection of steroids and carbohydrates apply equally well to these analyses. Without derivatization, only aminoacids with aromatic side-chain substituents are CD-active in the near-UV. Signals are generally weak, and enantiomeric purity measurements using polarimetry detection are not quantitative. Peptides and proteins have stronger rotatory powers with obvious potential for clinical analyses. Nevertheless, the major exploitations of these data are toward elucidating secondary and tertiary structural information in aqueous media (20, 48, 49). With respect to analytical applications, there is a larger role for these macromolecules as auxiliary or host substrates in determining low-molecular-weight substances that bind to the hosts in stereocontrolled ways, such as warfarin to human serum albumin for which FDCD is the preferred detector (50).

The ubiquitous involvement of these materials in chirality induction for the purposes of assaying small molecules, determining enantiomeric purities, quality control, and quantitative structure-activity relationships (QSAR) is reviewed later in this article. The detector that is common to all these applications is CD.

Natural Products in Plant Extracts

A special example of the analytical selectivity of CD is its ability to directly assay natural products in plant extracts. Because there are no reference standards for plant materials, an assay is deemed to be successful if the results lie within the expected compositional ranges for that material (51). Analyses are not fully quantitative. Direct assays have been described for tetrahydrocannabinol and cannabidiol in marihuana extracts (52); S-Nicotine in leaf extracts from tobacco and tobacco products (53); Pen-V extracted from a crude fermentation broth (36); vitamin C from a variety of whole fruits, fruit juices, and whole vegetables (40); reserpine alkaloids from Rauwolfia (54); pyrethroid insecticides (11); and amaryllidacea alkaloids (17); atropine from digitalis (24); and humulone from hops (24). Obviously, the most serious interferences would be from the absorbance by the plant pigments and light-scattering from suspended materials. The CD assays offer the advantage that the pigments are not fully extracted into the selected solvents, leaving the near-UV virtually transparent to absorption.

Exceptions to this last observation are encountered when the colored materials happen to be present in the same phase where, because of their excessive absorbances, the signal-to-noise ratio is decreased. This kind of complication was successfully handled in direct analytical assays devised for lysergic acid diamide (LSD) (55) and phencyclidine (PCP) in illicit drugs spiked with intensely colored dyes (56); for L-Cocaine, morphine, and methadone in the pharmaceutical product commonly referred to as Brompton's cocktails (57); and for D-Pseudoephedrine in children's Sudafed (37).

SUBSTRATES MADE CD-ACTIVE BY COLOR INDUCTION

From what has been learned from the near-UV studies, the selectivity and the sensitivity of CD detection are greatly enhanced if the CD-active absorption bands are shifted

from the wavelength range where the matrix absorbances are highest. With a few exceptions, the range of least interference is the visible. Wavelengths are shifted by using selective color or fluorescence derivatization reactions on chiral analytes as they exist in the matrix.

Color derivatizations can be broadly divided between reversible and irreversible reactions. Reversible reactions typically involve some kind of complex formation equilibrium. The color originates on the host and is imparted to the analyte on the formation of the complex. The combination of chirality and absorbance that produces CD activity is limited to only the complex. Any absorbance by uncomplexed host or any residual chirality on the uncomplexed analyte is not detected and are therefore not interfering. Because these are equilibrium reactions, the correlation between the experimental elliptically and the analyte concentration is nonlinear. An elegant and simple illustration of the capabilities of this kind of procedure is the determination of cholesterol in human gallstones in which association between the chiral cholesterol and colored bilirubin produced the CD-active complex (Fig. 7) (20, 50, 58, 59). Analogous reactions could be exploited for other naturally occurring pigments.

Other colored host molecules with obvious potential as reversible derivatizing agents are organic dyes and metal complexes of the first-row transition metals (26). Dyes, of course, are inherently strong absorbers, whereas transition metal complexes are not; however, the CD signals for the complexes are of similar intensities. Dyes are used as prosthetic binding groups in the CD analysis of peptides, proteins, and oligo- and polysaccharides, although more often, the object of the study is to discover stereochemical information about macromolecular structures in solution and at binding sites (6, 60). Their applications as hosts for the chemical analysis of simple molecules, oligopeptides, and nucleic acids using CD detection will ultimately follow.

An example of a colored metal complex as an analytical prosthetic reagent is alkaline Cu(II)-tartrate, which is used routinely for the determination of total-plasma protein. Detection is by absorbance. If racemic tartrate is replaced by the L-Enantiomer, the analytical reagent itself is CD-active. Exchanges of analyte ligands with the L-Tartrate induce significant changes from the CD spectrum of the host complex—changes that can be used not only for structural information about interactions occurring between the ligands in the first coordination sphere, but also as an analytical method selective toward the incoming ligand (26, 61, 62). The assay is a take-off from the analytical discrimination between neomycin B and neomycin C using ORD detection (26). The discrimination power of CD detection allied with the ligand exchange reaction on copper-L-tartrate was demonstrated for the amikacin, gentamycin, kanamycin, neomycin, and streptomycin antibiotics (26) (Fig. 8). The same host complex used to measure enantiomeric excesses or ratios (61, 62) in difficult-to-measure compositional ranges is addressed below.

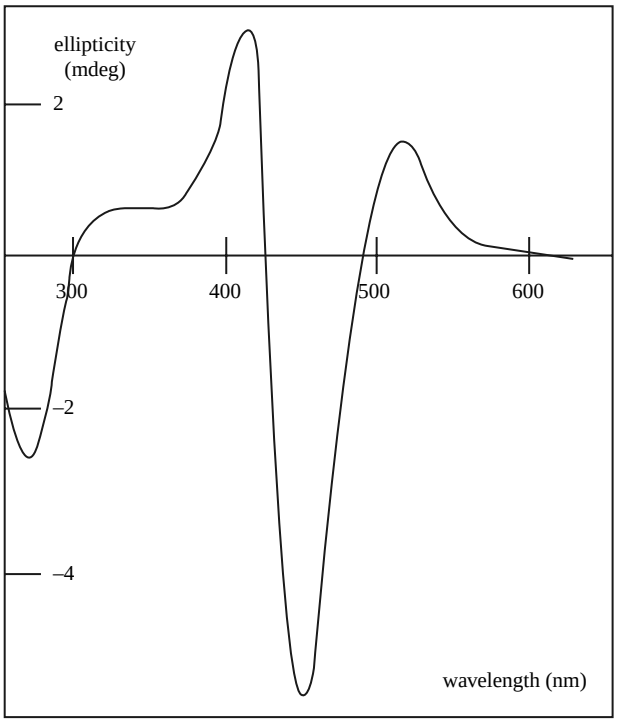

Fig. 7 Extrinsic (induced) CD spectrum for the complex formed between cholesterol and bilirubin in chloroform solution.

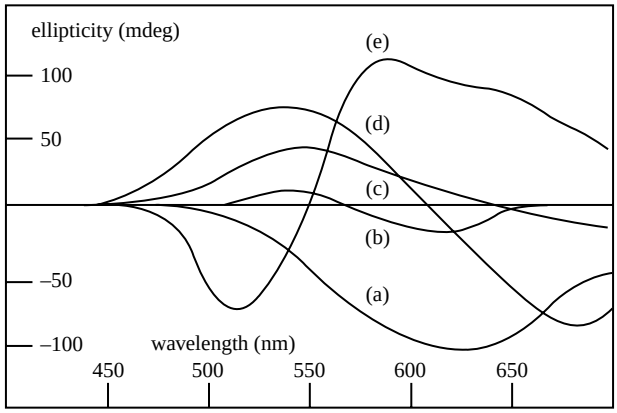

Fig. 8 CD spectra for (a) the chiral Cu(ll)-L-tartrate metal complex and for mixed complexes of this host with equimolar amounts of (b) kanamycin, (c) amikacin, (d) gentamycin, and (e) streptomycin.

The choice of an irreversible color-induction reaction requires more ingenuity and greater care in execution. If the extended molecular unsaturation required to produce the color is too exhaustive, the chiral centers could be systematically eliminated, and must be avoided. Reaction conditions are much more unfavorable. Reagents are generally toxic and corrosive, and reaction conditions are anhydrous, e.g., the measurement of plasma cholesterol using a modified Chugaev reagent (63).

SUBSTRATES MADE CD-ACTIVE BY CHIRALITY INDUCTION

Although the heading implies that the analyte is achiral, it does not have to be. Greater analytical selectivity can be engendered by specific mutual influences of intrinsic and extrinsic chirality properties. Chirality induction reactions are generally reversible. Many are used extensively in chiral liquid chromatography (12). Developments presented here are the changes toward chiroptical detection and the applications of direct spectral measurements. Optimal conditions are achieved when chirality resides only on the derivatizing agent, and the chromophore is limited to the analyte. On reaction, the CD activity is exclusive to the molecular complex. Potential interferences from host and guest are inconsequential. The best hosts are linear and cyclic forms of oligo- and polysaccharides, such as the oligomaltoses and the cyclodextrins, which absorb only in the far-UV. Oligomaltoses are more soluble but less selective than are cyclodextrins. Virtually any material that has been used as a stationary phase in chiral chromatography is a candidate for direct homogeneous association reactions, and many others will ultimately appear, e.g., cryptands, vesicles, micelles, peptides, proteins, enzymes, antibodies, and nucleic acids.

Complexation is an equilibrium process. It is recommended that the host molecule be kept in large compositional excess over the analyte, thereby maximizing the mass-action effect and complexing as much of the analyte as possible. The larger the formation constant, the straighter the correlation line between the experimental elliptically ΔA and the analyte concentration. The β- and γ-Cyclodextrins were used in exploratory investigations as analytical reagents (64) and to determine achiral forms of barbiturates (65), phenethylamines (64), benzodiazepin-2-ones (66), and phencyclidine and its analogs (5). Initial assays were done on prepared laboratory mixtures, but successful assays were also reported for secobarbital in seconal suppositories, meperidine in demerol dispensary products, and diazepam and flurazepam in pharmaceutical preparations (66).

The CD spectra for chiral complexes, formed by chirality induction on organic dyes by oligomaltoses and oligocelluloses, were used to elucidate the rotational direction of the helical structures of the hosts in aqueous media (60). CD spectra of chiral derivatives formed by the association of aromatic residues, such as 9-Anthroate and p-Hydroxycinnamate with analogous oligosaccharides, were used to probe the local stereochemistry of the ring linkages and conformational arrangements of adjacent groups in acyclic polyols (26).

Protein hosts, whose absorbance and CD spectra are dominated by intense signals in the far-UV range (190–230 nm) are appropriate choices for introducing chirality into any molecule that absorbs at longer wavelengths, e.g., associative complexations of proteins, enzymes, and/or oligopeptides with warfarin (50); bilirubin and its analogs (26, 50, 58, 59); and a few dye molecules (63). Beside the generation of analytical data, structural modifications at the active sites can be monitored over two spectral ranges: the far-UV, where the active group is the peptide bond, and the near-UV, where the absorber is the guest molecule. In addition, CD ought to be seriously considered as an alternative detector for immunoassays because the experimental selectivity might overcome some of the limitations associated with polyclonal antibodies.

DETERMINATION OF ENANTIOMERIC EXCESS IN PARTIAL RACEMIC MIXTURES

For all types of chemical analysis, the quality of the results ultimately relates to the chemical purity of the best available SRM. For naturally chiral substances, there is the additional more serious concern over what constitutes absolute enantiomeric purity. Not even mass spectroscopy, which provides assurance that a substance is chemically pure, can be used to report absolute enantiomeric purities. To actually report an enantiomeric purity higher than 99% is truly beyond the capability of current analytical methodology (20). As noted previously, the fact is that results are measured relative to an enantiopurity defined to be 100%. Chemical purities aside, the measurement of enantiomeric purity and enantiomeric excess is technically the same, the difference being the extent of racemization. There are only two experimental options, either enantiomeric separations or multivariate spectroscopic analyses, that involve either two distinct detectors or multiple-wavelength detection for a single detector, as noted above.

The newly described derivatization reactions fulfill the second option.

If the chosen derivatization reaction is chirality induction, a simple two-step process is to measure the CD spectrum for the underivatized partial racemic mixture, followed by a measure of the net spectrum after the addition of the derivatizing agent to the mixture. Fundamentally, the reaction is the simple competitive instantaneous complexation of the enantiomers with, for example, β-Cyclodextrin, in which two diastereoisomers are formed. These might have different formation constants, or different induced spectra, or both. Regardless, the result is a change in the original CD spectrum. The two unknown enantiomeric concentrations are calculated by solving the simultaneous equations that describe the additivity of the two enantiomers in the case of the original solution and the two diastereoisomers in the case of the derivatized solution. The method was used with limited success in the measurement of enantiomeric distribution for prepared nonracemic mixtures of R- and S-Nicotine (26). The poor results could in part be attributed to the spectral changes being very small.

More accurate results were achieved by exchanging both the enantiomers in prepared nonracemic mixtures of ephedrines and pseudoephedrines with the coordinated L-Tartrate ligand of the Cu(II)-L-Tartrate host complex dissolved in 0.10 *M* aqueous base (62). The first CD spectrum is that for the parent complex and the second for the mixed ligand complexes, where only one of the two bonded L-Tartrate ligands on Cu(II) is exchanged by either the (+)- or the (−)-ephedrine or pseudoephedrine enantiomers. The total signal is the sum of three terms, the CD signal from the decreased concentration of the parent complex, plus the CD signal from the 1:1 complex of Cu(II) with one L-tartrate and the (+) enantiomer, plus the CD signal from the 1:1 complex of Cu(II) with one L-Tartrate and the (−) enantiomer. With careful control of the reaction conditions, the detection limit for the enantiomeric purity was on the order of ±2%. In fact, changes in the CD signal on complexation was so large that almost equivalent results were more easily obtained using polarimetric measurements at only four wavelengths.

CD DETECTION/LIGAND EXCHANGE FOR ASSAYS OF PEPTIDES, OLIGOPEPTIDES, AND PROTEINS

With literally thousands of potential new drug substances in the combinatorial chemistry pipeline and the expanding emphasis on chiral drugs, the development of low-cost, routine quality-control procedures is becoming a priority in pharmaceutics and biotechnology. Ligand-exchange derivatization, coupled to CD detection, has the potential to do that for peptides, oligopeptides, and proteins.

Regulatory agencies have already set the standards for quality control (QC) for chiral drug substances. If it is a company's decision to market a chiral drug as a single enantiomer (the eutomer), the submission for regulatory approval must also include the equivalent chirality information for the other, nontherapeutic enantiomeric form (the distomer). An accurate determination of the enantiomeric purity of both forms is essential. Furthermore, chemical racemization will alter the eutomer to distomer ratio with time, diminishing the therapeutic property of the eutomer. Another very significant factor in QC, therefore, is to be able to accurately measure the rate of change of enantiomeric purity and decide when the meaningful therapeutic value of the eutomer has expired.

By replacing the L-tartrate ligand of the Cu(II)-derivatizing agent described above with D-histidine, a very selective host complex for ligand exchange was created (67–71). Much of the analytical selectivity accomplished by full spectrum visible-range CD detection is attributable to the specifics of the ligand–ligand interactions that ostensibly occur within the first coordination sphere of the complex. The extent of the selectivity that is accomplished for peptides and proteins is extraordinarily high (Fig. 9).

It was also determined that the CD spectral changes are extremely sensitive to changes in the amino acid sequence among residues that are far removed from the binding sites. A case in point is the individualized CD spectral changes for the exchange of D-histidine with human, human LysPro, porcine, and bovine insulin forms, all of which are 51 amino acid residue proteins

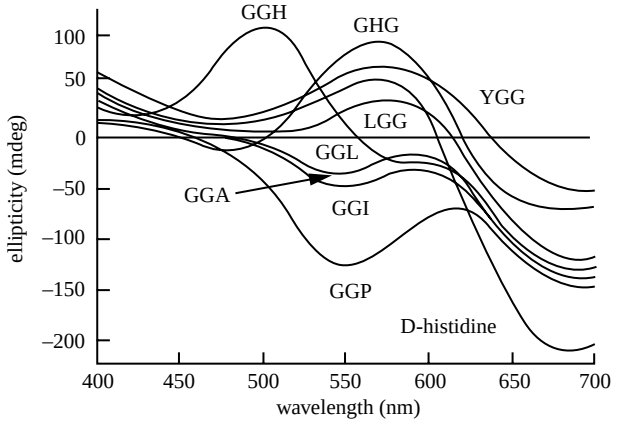

Fig. 9 CD spectra for the host Cu(II)-D-histidine spectrum and the mixed complexes with a series of tripeptides.

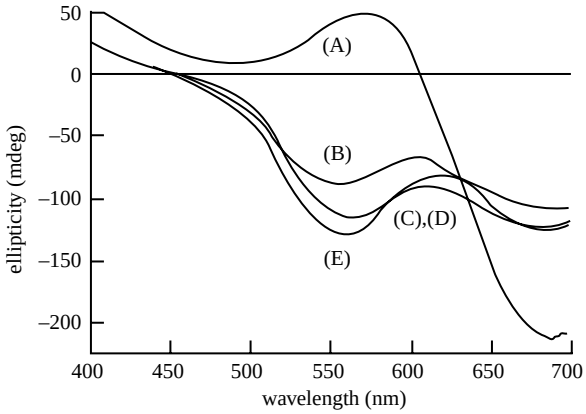

Fig. 10 CD spectra for (A) the Cu(II)-D-histidine host complex and for the mixed ligand complexes with (B) bovine insulin, (C) human insulin, (D) porcine insulin, and (E) human LysPro insulin.

(70). It is a known fact that proteins bind to Cu(II) ion in aqueous base by first substitution via the *N*-atom at the amine terminus (72). Chelation is completed by second and third substitutions into the metal first coordination sphere through the *N*-atoms of the first and second peptide functional groups. Because all four insulins have identical initial sequences, the observed CD spectral changes on ligand exchange are caused by some other structural variations. The only differences in the insulin residue sequences occur either at or adjacent to the acid terminus of the B-chain. Human insulin differs from human LysPro by the simple exchange of lysine and proline residues at positions B-28 and B-29. Porcine and human insulins differ by one residue, alanine for threonine, at position B-30 (70). The only explanation that accounts for this is that the metal ion is enclosed by an extensive, flexible, chiral, three-dimensional architecture and that subtle differences in the conformational properties of the enclosure bring different residues into the realm of the ligand to metal ion electronic transitions. With the D-Histidine kept in very large excess over the insulin analytes, there is a good quantitative linear correlation of the CD signal with concentration. What many researchers may have failed to realize in drug modeling is that when two chiral molecules interact, the stereochemical conformations of both change, and these changes may be just as significant in the mechanism of drug action as the absolute conformations of the participants themselves.

In other tests of the ligand exchange/CD detection assay procedure, it was demonstrated that 51 of a total of 53 di- and tripeptides could be uniquely identified by the character of the changes in the CD spectra for the mixed Cu(II)-peptide-D-histidine complexes (67, 68, 70). A series of 19 neuropeptides was the focus of another investigation (69, 71). Neuropeptides are invariably chiral molecules and, because of the peptide bond, are CD-active. Once again, all but two of the analogs were individually distinguishable by the CD spectra for the mixed ligand complexes. For the two exceptions, ICI 174,864 and PLO17, the terminal amine group is substituted and, as such, it is not competitive with D-histidine in binding to the Cu(II) ion. In other words, substitution did not occur. To determine whether the CD spectra had characteristic properties that could be associated with the structure of first coordination spheres of the metal complexes, a Y-correlation matrix of the CD data was subjected to principal component analysis (PCA). All of the spectra can be accounted for mathematically by only four factors (71). The spectral data for each neuropeptide analyte can be graphically represented by a single point in an X–Y plot of the first two principal components, PC1 and PC2. When the points are subjected to a hierarchical clustering algorithm, the neuropeptides

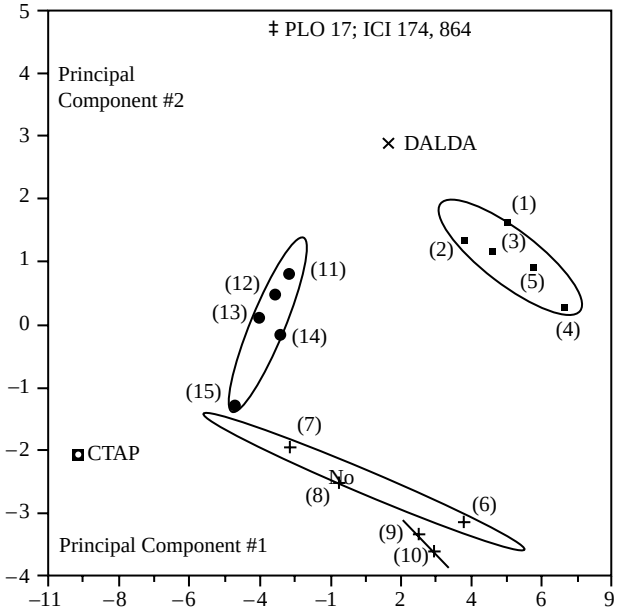

Fig. 11 Cluster plot of the first and second principal components derived by PCA from spectral data. The δ-receptor cluster covers (1) DTLET, (2) DSLET, (3) DADLE, (4) a^2-Leu^5-enkephalin, and (5) DPDPE. The μ-receptor cluster includes (6) DAGO, (7) Met^5-enkephalin, and β-endorphin. The alternate δ-receptor cluster is composed of (9) Leu^5-enkephalin and (10) Leu^5-enkephalin amide. The κ-cluster of the dynorphins contains (11) B(1–13), (12) A(1–13); (13) A(1–9); (14) A(1–11); and (15) A(1–13) amide. No receptor preference was reported for PLO 17 or ICI 174 864.

are observed to aggregate according to their preferences for the δ, μ, or κ protein receptors (Fig. 11). This calibration model has the potential to become a prototypical-predictive in vitro model for correlating CD spectroscopic data with quantitative structure-activity relationships (QSAR) and, on further substantiation, may become a viable procedure for new drug forms (71). Despite the fact that the system is a mixed equilibrium reaction, in quantitative terms, a strong linear correlation exists between PC1 and analyte concentrations.

A recurring chiral property in many of the neuropeptides is the presence of at least one D-enantiomeric residue, e.g., natural and designer enkephalins such as DALDA, DAGO, and DPDPE. Locating the position of the D-form in a sequence is a challenging endeavor that is being systematically studied on two series of model penta- and hexapeptides. The role of D-enantiomeric forms in biotechnology drug substances is a very real interest and more especially in view of the recent recognition of the existence of D-Serine, which functions as neurotransmitter in mammalian brain tissue (73). The D-Enantiomer is synthesized in the brain from the natural L-Form catalyzed by serine racemase.

SUMMARY

The intent of this article was to demonstrate, based on a wealth of relatively new experimental data, that there is sufficient analytical selectivity and sensitivity to accept polarimetry and CD as viable and easy-to-use analytical detection methods. In contrast to other detectors, they provide the capability of making direct analytical assays after a sample work-up that is a simple solvent extraction and of measuring enantiomeric purities in the ranges specified by the FDA for the process and quality control for new chiral drug substances. In the future, broader applications will be developed, especially as the current analytical emphasis turns toward nucleic acid protein interactions and the CD properties of intact single cells.

REFERENCES

1. Lowry, T.M. *Optical Rotatory Power*; Dover Publications: New York, 1935.
2. Djerrasi, C. *Optical Rotatory Dispersion and Circular Dichroism in Organic Chemistry*; McGraw-Hill: New York, 1960.
3. Charney, E. *The Molecular Basis of Optical Activity. Optical Rotatory Dispersion and Circular Dichroism*; Wiley: New York, 1979.
4. Schnatzke, G. *Optical Rotatory Dispersion and Circular Dichroism in Organic Chemistry*; Heyden: London, 1967.
5. Crabbe, P. *ORD and CD in Chemistry and Biochemistry. An Introduction*; Academic Press: New York, 1972.
6. Harada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy: Exciton Coupling in Organic Stereochemistry*; University Science Books: Mill Valley, CA, 1983.
7. Cotton, A. Compt. Rend. **1895**, *120*, 989.
8. Bobbitt, D.R. Analytical Applications of Circular Dichroism. *Technical Instrumentation in Analytical Chemistry*; Purdie, N., Brittain, H.G., Eds.; Elsevier: Amsterdam, 1994; 14, Chap. 2.
9. Sutherland, J.C. *Circular Dichroism and the Conformational Analysis of Biomolecules*; Fasman, G.D., Ed.; Plenum Press: New York, 1996.
10. Synovec, R.E.; Yeung, E.S. Fluorescence Detected Circular Dichroism as a Detection Principle in High-Performance Liquid Chromatography. J. Chromatogr. **1986**, *368*, 85–93.
11. Lloyd, D.K.; Goodall, D.M.; Scrivener, H. Diode-Laser-Based Optical Rotation Detector for High-Performance Liquid Chromatography and On-Line Polarimetric Analysis. Anal. Chem. **1989**, *61*, 1238–1243.
12. Christensen, P.L.; Yeung, E.S. Fluorescence-Detected Circular Dichroism for On-Column Detection in Capillary Electrophoresis. Anal. Chem. **1989**, *61*, 1344–1347.
13. Yeung, E.S.; Synovec, R.E. Detectors for Liquid Chromatography. Anal. Chem. **1986**, *58*, 1237–1256.
14. Takakuwa, T.; Kurosu, Y.; Sakayanagi, N.; Kaneuchi, F.; Takeuchi, N.; Wada, A.; Senda, M. Direct Combination of a High Performance Liquid Chromatograph and a Circular Dichroism Spectrometer for Separation and Structural Analysis of Proteins. J. Liq. Chromatogr. **1987**, *10*, 2759–2769.
15. Westwood, S.A.; Games, D.E.; Sheen, L. Use of Circular Dichroism as a High-Performance Liquid Chromatography Detector. Chromatography **1981**, *204*, 103–107.
16. Mannschreck, A. On-Line Measurement of Circular Dichroism Spectra During Enantioselective Liquid Chromatography. Trends. Anal. Chem. **1993**, *12*, 220–225.
17. Boehme, W.; Wagner, G.; Oehme, U. Spectrophotometric and Polarimetric Detectors in Liquid Chromatography for the Determination of Enantiomer Ratios in Complex Mixtures. Anal. Chem. **1982**, *54*, 709–711.
18. Meinard, C.; Bruneau, P.; Perronett, J. High-Performance Liquid Chromatograph Coupled with Two Detectors: A UV Spectrometer and a Polarimeter. J. Chromatogr. **1985**, *349*, 109–116.
19. Ward, T.J.; Armstrong, D.W. *Chromatographic Science Series Chromatographic Chiral Separations*; Zief, M., Crane, L.J., Eds.; Marcel Dekker, Inc.: New York, 1988; 40.
20. Zukowski, J.; Tang, Y.; Berthod, A.; Armstrong, D.W. Investigation of a Circular Dichroism Spectrophotometer as a Liquid Chromatography Detector for Enantiomers: Sensitivity, Advantages, and Limitations. Anal. Chim. Acta. **1992**, *258*, 83–92.

21. Kudo, K.; Ajima, K.; Sakamoto, M.; Saito, M.; Morris, S.; Castiglioni, E. New Circular Dichroism (CD) Based Detection for Monitoring Chiral Compounds in HPLC. Chromatography **1999**, *20*, 59–64.
22. Horvath, P.; Gergely, A.; Noszal, B. Determination of Enantiomeric Purity by Simultaneous Dual Circular Dichroism and Ultraviolet Spectroscopy. Talanta **1997**, *44*, 1479–1485.
23. Lodevico, R.G.; Bobbitt, D.R.; Edkins, T.J. Determination of Enantiomeric Excess Using a Chiral Selective Separation Mode and Polarimetric Detection. Talanta **1997**, *44*, 1353–1363.
24. Purdie, N.; Swallows, K.A. Analytical Applications of Polarimetry, ORD, and CD. Anal. Chem. **1989**, *61*, 77–86.
25. Purdie, N.; Swallows, K.A.; Murphy, L.B.; Purdie, R.B. Circular Dichroism. II. Analytical Applications. Trends Anal. Chem. **1990**, *9*, 136–142.
26. Purdie, N. Analytical Applications of Circular Dichroism. *Technical Instrumentation in Analytical Chemistry*; Purdie, N., Brittain, H.G., Eds.; Elsevier: Amsterdam, 1994; 14, Chap. 8.
27. Martens, H.; Naes, T. *Multivariate Calibration*; Wiley: New York, 1989.
28. Crone, T.A.; Purdie, N. Circular Dichroism Spectra of Opium Alkaloids in Aqueous Media. Anal. Chem. **1981**, *53*, 17–21.
29. Bowen, J.M.; Crone, T.A.; Hermann, A.O.; Purdie, N. Circular Dichroism Spectra of Opium Alkaloids in a Cholesteric Liquid Crystalline Solvent System. Anal. Chem. **1980**, *52*, 2436–2440.
30. Bowen, J.M.; Purdie, N. Circular Dichroism Spectra of Opium Alkaloids in the Solid State. Anal. Chem. **1980**, *52*, 573–575.
31. Bowen, J.M.; Crone, T.A.; Kennedy, R.K.; Purdie, N. Determination of Heroin by Circular Dichroism. Anal. Chem. **1982**, *54*, 66–68.
32. Han, S.M.; Purdie, N. Simultaneous Determination of Opiates by Circular Dichroism. Anal. Chem. **1986**, *58*, 113–116.
33. Potapov, V.M.; Demianovich, V.M.; Terentiev, A.P. Spectropolarimetric Analysis. IV. Polarimetric Analysis of Ephedrines and Pseudoephedrines in Their Mixtures. Zh. Anal. Khim. **1964**, *19*, 254–257.
34. Bowen, J.M.; Crone, T.A.; Head, V.L.; McMorrow, H.A.; Kennedy, R.K.; Purdie, N. Circular Dichroism: An Alternate Method for Drug Analysis. J. Forensic. Sci. **1981**, *26*, 664–670.
35. Mitscher, L.A. *The Chemistry of the Tetracycline Antibiotics*; Marcel Dekker, Inc.: New York, 1978; 13.
36. Purdie, N.; Swallows, K.A. Direct Determination of Beta-Lactam Antibiotics by Circular Dichroism. Anal. Chem. **1987**, *59*, 1349–1351.
37. Engle, A.R., Ph.D. Dissertation. Oklahoma State University: Stillwater, OK, 1993.
38. Han, S.M.; Purdie, N. Determination of Stereoisomers by Circular Dichroism. Anal. Chem. **1986**, *58*, 455–458.
39. Purdie, N. Determination of Drugs of Abuse and their Stereoisomers by Circular Dichroism. Forensic. Sci. Rev. **1991**, *3*, 1–16.
40. Purdie, N.; Swallows, K.A. Direct Determination of Water Soluble Vitamins by Circular Dichroism. J. Agr. Food Sci. **1991**, *39*, 2171–2175.
41. Potapov, V.M.; Demianovich, V.M.; Rakovska, R.S. Analytical Applications of Spectropolarimetry. Zh. Anal. Khim. **1984**, *39*, 983–996.
42. Gergely, A. A Review of the Application of Chiroptical Methods to Analytical Chemistry. J. Pharm. Biomed. Anal. **1989**, *7*, 523–541.
43. Johnson, W.C., Jr. The Circular Dichroism of Carbohydrates. Adv. Carbohydr. Chem. Biochem. **1987**, *45*, 73–124.
44. Hayward, L.D.; Angyal, S.J. A Symmetry Rule for the Circular Dichroism of Reducing Sugars, and the Proportion of Carbonyl Forms in Aqueous Solutions Thereof. Carbohydr. Res. **1977**, *53*, 13–20.
45. Domard, A. pH and CD Measurements on a Fully Deacetylated Chitosan. Int. J. Biol. Macromol. **1987**, *9*, 333.
46. Kuo, J.C.; Yeung, E.S. Determination of Carbohydrates in Urine by High-Performance Liquid Chomatography and Optical Activity Detection. J. Chromatogr. **1981**, *223*, 321–329.
47. Kimura, A.; Chiba, S.; Yoneyama, M. A Simple and Rapid Determination of Ketoses by Circular Dichroism. Carbohydr. Res. **1988**, *175*, 17–23.
48. Fasman, G.D. *Circular Dichroism and the Conformational Analysis of Biomolecules*; Plenum Press: New York, 1996.
49. Jirgensons, B. *Optical Activity of Proteins and Other Macromolecules*, 2nd Ed.; Springer Verlag: New York, 1973.
50. Thomas, M.P.; Patonay, G.; Warner, I.M. Fluorescence-Detected Circular Dichroism Studies of Serum Albumins. Anal. Biochem. **1987**, *164*, 466–473.
51. Souci, S.W.; Fachmann, W.; Kraut, H. *Food Composition and Nutrition Tables*, 2nd Ed.; Wissenschaflliche Verlagsgesellschafl mbH: Stuttgart, 1981.
52. Han, S.M.; Purdie, N. Determination of Cannabinoids by Circular Dichroism. Anal. Chem. **1985**, *57*, 1068–1072.
53. Atkinson, W.M.; Han, S.M.; Purdie, N. Determination of Nicotine in Tobacco by CD Spectropolarimetry. Anal. Chem. **1984**, *56*, 1947–1950.
54. Purdie, N.; Swallows, K.A. Determination of Reserpine by Circular Dichroism. Pharm. Res. **1989**, *6*, 521–524.
55. Bowen, J.M.; McMorrow, H.A.; Purdie, N. Determination of LSD in Drug Confiscates by Circular Dichroism Spectropolarimetry. J. Forensic. Sci. **1982**, *27*, 822–826.
56. Bowen, J.M.; Purdie, N. Identification of Cocaine and Phencyclidine by Solute-Induced Circular Dichroism. Anal. Chem. **1981**, *53*, 2239–2242.
57. Atkinson, W.M.; Bowen, J.M.; Purdie, N. Determination of Brompton's Cocktails by CD. J. Pharm. Sci. **1984**, *173*, 1827–1828.
58. Blauer, G. Complexes of Bilirubin with Proteins. Biochim. Biophys. Acta. **1986**, *884*, 602–605.

59. Blauer, G. Optical Activity of Bile Pigments. Israel J. Chem. **1983**, *23*, 201–209.
60. Engle, A.R.; Hyatt, J.A.; Purdie, N. Induced Circular Dichroism Study of the Aqueous Solution Complexation of Cello-Oligosaccharides and Related Polysaccharides with Aromatic Dyes. Carbohydr. Res. **1994**, *265*, 181–195.
61. Engle, A.R.; Lucas, E.A.; Purdie, N. Determination of Enantiomers of Ephedrine Mixtures by Polarimetry. J. Pharm. Sci. **1994**, *83*, 1310–1314.
62. Engle, A.R.; Purdie, N. Determination of Enantiomeric Purities Using CD/CD Detection. Anal. Chim. Acta. **1994**, *298*, 175–182.
63. Purdie, N.; Murphy, L.H.; Purdie, R.B. Serum Cholesterol: A Direct Measure of the Low Density Fractions. Anal. Chem. **1991**, *63*, 2947–2951.
64. Han, S.M.; Atkinson, W.M.; Purdie, N. Solute Induced CD: Drug Discrimination by Cyclodextrin. Anal. Chem. **1984**, *56*, 2827–2830.
65. Han, S.M.; Purdie, N. Cyclodextrin Complexation of Barbiturates in Aqueous Solution. Anal. Chem. **1984**, *56*, 2825–2827.
66. Han, S.M.; Purdie, N.; Swallows, K.A. Determination of the Benzodiazepin-2-Ones by Circular Dichroism. Anal. Chim. Acta **1987**, *197*, 57–64.
67. Purdie, N.; Province, D.W. Algorithms for the Quantitative Validation of Chiral Properties of Peptides. Chirality **1999**, *11*, 546–553.
68. Purdie, N.; Province, D.W.; Johnson, E.A. Tripeptide Discriminations Using Circular Dichroism Detection. J. Pharm. Sci. **1999**, *88*, 715–721.
69. Purdie, N.; Province, D.W.; Johnson, E.A. A Convenient Assay Method for the Quality Control of Peptides and Proteins. J. Pharm. Sci. **1999**, *88*, 1242–1248.
70. Purdie, N.; Province, D.W.; Layloff, T.P.; Nasr, M.M. Algorithms for Validating Chiral Properties of Insulins. Anal. Chem. **1999**, *7*, 3341–3346.
71. Purdie, N.; Province, D.W. Clustering of CD Spectral Data as a Prototype QSAR Model for Neuropeptides. J. Pharm. Sci. **1999**, *88*, 1249–1253.
72. Sigel, H.; Martin, R.B. Coordinating Properties of the Amide Bond. Stability and Structure of Metal Complexes of Peptides and Related Ligands. Chem. Rev. **1982**, *82*, 384–426.
73. Snyder, S.H.; Wolosker, H.; Blackshaw, S. Serine Racemase: A Glial Enzyme Synthesizing D-Serine to Regulate Glutamate-*N*-methyl-D-Aspartate Neuro-Transmission. Proc. Natl. Acad. Sci. USA **1999**, *96*, 13409–13414.